Serial No. 10/791,628
Filing Date: March 1, 2004

RECEIVED
CENTRAL FAX CENTER
JUN 07 2007

REMARKS

Claims 1-5, 8-10, 19-40, 44-47 and 56-73 are pending; claims 11-18, 48-55 and 74-145 are withdrawn; claims 1, 8, 10, 19, 34, 35, 37, 44, 45, 59, 60 and 72 have been amended; claims 6, 7, and 41-43 have been cancelled.

I. Objections to the disclosure

The Examiner objected to the use of the abbreviation "SBL", and requests that this abbreviation for "subtilisin" be defined in the specification at page 4, line 9 (see pages 2-3 of the Office Action).

The Examiner also requested that a verb be used in the sentence on page 18, line 5 (see page 3 of the Office Action).

Applicants have amended the specification accordingly to address the Examiner's objections, and request that the objections made to the specification be withdrawn.

II. Rejection under 35 U.S.C. §112, second paragraph

The Examiner has rejected claims 19, 34-35, 59-60 and 72 under 35 U.S.C. §112, second paragraph, as being indefinite. Specifically, the Examiner indicates that claim 19 lacks antecedent for the word "target", claim 34 is confusing in the recitation of "wherein targeting", claims 35 and 72 are confusing in the recitation of "-thioethyl", and claims 59-60 for missing the article "a" in the phrase "is component".

Applicants have amended the claims according to the suggestions made by the Examiner, and request that the rejection of the claims under 35 U.S.C. §112, second paragraph, be withdrawn.

III. Rejection under 35 U.S.C. §112, first paragraph

A. The Examiner has rejected claims 10 and 47 under 35 U.S.C. §112, first paragraph, for failing to comply with the enablement requirement.

In particular, the Examiner states that claims 10 and 47 refer to a *Bacillus lentus* subtilisin for which a sequence is not provided in the disclosure. The Examiner indicates that the claims refer to particular residue positions in a Bacillus lentus subtilisin, while

"there is no sequence disclosure in the instant application and therefore one of ordinary skill in the art reading this specification would not know what particular sequence was being referred to."

Reference to the Bacillus lentus subtilisin is provided in the disclosure at least in the paragraph at page 47, beginning at line 26, which references U.S. patent 5,185,258. U.S. patent 5,185,258 provides the Bacillus lentus amino acid sequence in Figure 4 and describes the cloning of the subtilisin into a vector pGG36, from which the subtilisin protein can be expressed (for example see Example 1 of U.S. Patent 5,185,258).

Therefore, claims 10 and 47 are enabled, and Applicants request that the Examiner withdraw the rejection of claims 10 and 47 under 35 U.S.C. §112, first paragraph.

B. The Examiner has rejected claims 1-10, 19-47 and 56-73 under 35 U.S.C. §112, first paragraph, for failing to comply with the enablement requirement.

At page 4 of the Office Action, the Examiner submits that one of ordinary skill in the art would not understand the metes and bounds of the term "cognate ligand" as recited in claims 1, 25 and 62.

Applicants submit that the term "cognate ligand" is well understood by one of skill in the art as meaning a molecule that can specifically bind another molecule. A definition of a "cognate ligand" is provided in the specification at page 8, line 30. In the context of the present invention, the target molecule recognizes and binds a cognate ligand, which when incorporated into a catalytic antagonist as the targeting molety, can specifically bind the target molecule to degrade it.

At page 4 of the Office Action, the Examiner stated that "Reference is made throughout the specification to "GG36-WT", and requested that "Applicants should define this term, showing where in the prior art this term is used".

The Examiner correctly presumes that the term refers to a wild-type subtilisin, and Applicants have amended the specification accordingly. Tables 2, 30, and 31 provide results that compare the activity of mutant enzymes to "GG36WT". Reference is also made to GG36 in the prior art, for example, GG36 is disclosed in U.S. patent 5,185,258 disclosed in the present specification at page 47, line 26. U.S. patent

5,185,258 provides the sequence for GG36 in Figure 4, and refers to the plasmid encoding the amino acid sequence as "pGG36 from Bacillus lentus (ATCC 21536)" in Example 1, and further references the amino acid sequence as "The amino acid sequence for this subtilisin is the same as that disclosed for subtilisin 309 in PCT Publication No. 89/06279". Further, GG36 wild type is referred to in U.S. Patent 6,946,280 filed on March 29, 1996.

Thus, the GG36 sequence was known in the art at the time of filing of the present application.

At pages 4 and 5 of the Office Action, the Examiner indicated that the claims are not enabled because the examples provided in the specification do not support "that after the targeting moiety degrades the target molecule, it is released to bind another target molecule, as required by the instant claims". In particular, the Examiner refers to Example 3 and Example 4.

Example 3 describes an experiment in which the concentration of catalytic antagonist exceeds that of the target molecule. In light of the data provided in Example 3, the Examiner concluded that "with the subtilisin molecule being in [access] excess it could just bind and degrade the HLADH and remain bound to it and the same data be obtained". The Examiner further argues that Table 11 (Example 4) "does not refute this assertion as after 3 hours the percentage activity with the S166C-pyrazole was greater than the same time in Table 7" (Example 3).

Applicants submit that while the experiment in Example 3 may not necessarily prove the binding, degradation, release and further binding of the targeting moiety, it does not disprove it. Furthermore, the results provided in Table 11 of Example 4 show that at 20 hours the % HLADH is reduced to 17%. Example 4 describes an experiment in which the ratio of target molecule to targeting moiety is 4:1. Table 11 shows that at 20 hours the % HLADH is reduced to 17%. In presence of excess (4 fold) target molecule to targeting moiety, this reduction can be attributed to a repeating cycle of binding, degradation, release and further binding of the targeting moiety, as required by the claims.

Thus, Applicants submit that the specification enables the claims, and respectfully request that the rejection be withdrawn.

In the second paragraph at page 5 of the Office Action the Examiner questions the meaning of "amidase kinetics" and "ESMS" in Tables 2 and 30, and "amidase activity" in Table 44, stating that "Exactly what these labels represent and the significance of them in measuring the effectiveness of pyrazole-CMM and biotin-CMM...is not understood".

"ESMS" is an acronym for "electron spray mass spectrometry", and is a term well known in the art. For example, please see http://209.85.135.104/search?q=cache:rr5faZAXqQUJ:acronyms.thefreedictionary.com/ESMS+ESMS+definition&hl=en&ct=clnk&cd=1&gl=us

Tables 2 and 30 respectively provide the results of the test of the activity of a subtilisin, whether wild-type or mutant, when the subtilisin is chemically modified to contain a pyrazole or a biotin group. The amidase kinetics provided in Table 30 are the results of the test of the activity of the biotin-modified enzymes in the presence of the substrate suc-AAPFpNA (Suc-Ala-Ala-Pro-Phe-pnitroanilide), which is used to determine the amidase activity of the modified enzymes. The "amidase activity" given in Table 44, is the activity of the biotin-modified enzymes determined using antibodies as substrate.

The Examiner also indicates that Tables 30 and 31 contain three columns each having two sets of numbers per line, and suggests that should the second set of numbers reflect the margin of error, that the Tables be amended accordingly. Applicants thank the Examiner for suggesting that the Tables be amended to the format given in Table 2, and have amended the specification accordingly.

At page 6 of the Office Action, the Examiner stated that "suc-AAPF-SBn" is used" but "apparently it is not disclosed what suc-AAPF-SBn is so that the significance of this assay cannot be ascertained". suc-AAPF-SBn is the ester substrate succinyl-Ala-Ala-Pro-Phe-S-benzyl used to measure the esterase activity of the modified enzymes.

Applicants submit that a person skilled in the art would have known the meaning of the term at the time of filing of the application, and enclose herewith a copy of the reference by Estell at al, which supports Applicants' assertion.

C. The Examiner has rejected claims 1-7, 19-28, 37-42, 44 and 56-65 under 35 U.S.C. §112, first paragraph, for failing to comply with the enablement requirement.

In particular, the examiner states that all "the embodiments in the instant specification [that] have been shown to be operable using mutants of subtilisin and that one of ordinary skill in the art is not taught by the specification how to make any other catalytic antagonists".

While Applicants must respectfully disagree, in order to expedite prosecution and yet without acquiescing to the Examiner's arguments, Applicants have amended independent Claims 1 and 37, without prejudice. Claims 1 and 37, as amended are directed to a subtilisin-type serine hydrolyse. Support for the amendment can be found, for example, in the paragraph beginning at page 11, line 19 and the paragraph beginning at page 13, line 13.

In light of the foregoing, the rejection of Claim 1-7, 19-28, 37-42, 44 and 56-65 under 35 U.S.C. §112, first paragraph, should be withdrawn.

IV. Rejection under 35 U.S.C. §102

The Examiner has rejected Claims 1 and 37 under 35 U.S.C. §102(b) as being anticipated by Chandrasegaran.

Applicants respectfully traverse the rejection.

Chandrasegaran teach "DNA segments encoding the recognition and cleavage domains of the FokI restriction endonuclease", and "hybrid restriction enzymes comprising the nuclease domain of the FokI restriction endonuclease linked to a recognition domain of another enzyme". The enzyme or hybrid enzymes taught by Chandrasegaran contain protein sequences that define the cleavage and sequence recognition domains. Chandrasegaran does not teach a catalytic antagonist comprising a subtilisin-type serine hydrolase to which a targeting moiety has been attached, as required by claims 1 and 37, as amended.

Accordingly, reconsideration and withdrawal of the rejection are proper and respectfully requested.

The Examiner also cited Davis et al., and Epenetos et al. as of interest. As discussed above, Applicants have explained that the specification indeed teaches that the catalytic antagonists bind and degrade a target molecule, to then bind and degrade another target molecule.

Applicants submit that neither Davis et al. nor Epenetos et al. anticipate the instant claims.

RECEIVED
CENTRAL FAX CENTER
JUN 07 2007

Serial No. 10/791,628
Filing Date: March 1, 2004

CONCLUSION

Applicants believe the pending claim is in condition for allowance and issuance of a formal Notice of Allowance at an early date is respectfully requested. If a telephone conference would expedite prosecution of this application, the Examiner is invited to telephone the undersigned at (650) 846-7636.

This paper is accompanied by a Petition to Revive the present application. The Commissioner is authorized to charge any fees that may be required in connection with this submission and to credit any overpayments to Deposit Account No. 07-1048 (Attorney Docket No. GC571-2-C1).

Respectfully submitted,

Dated: June 7, 2007

Elena Quertermous
Reg. No. 47,873

Danisco US Inc., Genencor Division
925 Page Mill Road
Palo Alto, CA 94304-1013
Tel.: (650) 846-7500, Ext. 7636
Fax: (650) 845-6504

Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 260, No. 11, Issue of June 10, pp. 6518–6521

Engineering an Enzyme by Site-directed Mutagenesis to Be Resistant to Chemical Oxidation*

(Received for publication, February 4, 1985)

David A. Estell‡, Thomas P. Graycar‡, and James A. Wells§

From the ‡Department of Enzymology, Genencor, Inc. and the §Department of Biocatalysis, Genentech, Inc., South San Francisco, California 94080

Site-directed mutagenesis can be employed to alter activity critical residues in proteins which are susceptible to chemical oxidation. Previous studies have implicated methionine 222 as a primary site for oxidative inactivation of subtilisin (Stauffer, C. E., and Etson, D. (1969) *J. Biol. Chem.* 244, 5333–5338). Because of uncertainties in predicting which amino acid would be the optimal substitute for methionine 222, we prepared all 19 amino acid substitutions at this site in the cloned subtilisin gene using a cassette mutagenesis method (Wells, J. A., Vasser, M., and Powers, D. P. (1985) *Gene* (*Amst.*), in press). Mutant enzymes were expressed in *Bacillus subtilis* and were found to vary widely in specific activity. Mutants containing nonoxidizable amino acids (*i.e.* Ser, Ala, and Leu) were resistant to inactivation by 1 M H_2O_2, whereas methionine and cysteine enzymes were rapidly inactivated. These studies demonstrate the feasibility of improving oxidative stability in proteins by site-directed mutagenesis.

One of the primary sources of protein instability is their susceptibility to oxidation and subsequent inactivation or denaturation (for review, see Brot and Weissbach, 1983). This is especially true for proteins containing methionine, cysteine, or tryptophan residues in or around the active site. While methionine sulfoxide in proteins can be reduced *in vivo* (Brot *et al.*, 1981), industrial applications of enzymes and proteins can be hampered by oxidative inactivation. It would therefore be useful to investigate the functional consequences of replacing activity critical residues which are sensitive to oxidation. Subtilisin, a serine protease from *Bacillus* species, contains an invariant methionine residue at position 222 (Markland and Smith, 1971). Treatment of the enzyme with H_2O_2 leads to inactivation that correlates directly with the production of methionine sulfoxide at position 222 (Stauffer and Etson, 1969).

We have cloned and expressed the gene for *Bacillus amyloliquefaciens* subtilisin (Wells *et al.*, 1983). Site-directed mutagenesis methods (Wallace *et al.*, 1981; Zoller and Smith, 1982) permit the replacement of methionine 222 with any amino acid. Although the three-dimensional structure of subtilisin is known (Wright *et al.*, 1969; Drenth *et al.*, 1972), it is not obvious from the existing data base which nonoxidizable residue would be the optimal substitute for methionine 222 to retain enzymatic activity. The phenylmethylsulfonyl fluoride-inhibited subtilisin structure of Wright et al. (1969), shown in Fig. 1, indicates that the methionine is largely buried among the side chains of tyrosine 217, histidine 64, histidine 67, and the main chain atoms 217–218. To complicate matters, the diisopropyl fluorophosphate-inhibited subtilisin structure of Drenth *et al.* (1972) shows methionine 222 to be largely solvent-exposed. Methionine 222 is next to the catalytic site serine 221 and sits at the amino-terminal end of an α-helix in the molecule.

Predictions based on homologous exchanges of amino acids in related proteins (Dayhoff *et al.*, 1978) would suggest leucine or valine as the most homologous substitution for methionine. However, these homologous exchange data are most often generated from sites which have little or no apparent functional significance. Because of structural uncertainties and the implication that substitution of methionine 222 could have significant functional consequences, we elected to make many substitutions at codon 222 and screen for a more oxidatively stable mutant.

MATERIALS AND METHODS

Production of all 19 amino acid substitutions at codon 222 of the subtilisin gene employed a cassette mutagenesis[1] strategy previously described (Wells *et al.*, 1985). Briefly, silent restriction sites (*i.e.* *Pst*I and *Kpn*I) were introduced into the *B. amyloliquefaciens* subtilisin gene (Wells *et al.*, 1983) by site-directed mutagenesis. The *Pst*I and *Kpn*I sites produced were unique and were designed to flank closely the target codon 222. Digestion of the plasmid with *Pst*I and *Kpn*I produced a 25-base pair gap that removed the sequence including codon 222. Duplex synthetic oligonucleotide cassettes were ligated into the gap between the restriction sites. The cassettes were designed to restore the coding sequence in the gap and to introduce an altered codon at position 222.

Bacillus subtilis strain BG2036, which contains deletions in the structural genes for neutral protease and subtilisin (Yang *et al.*, 1984), was transformed with plasmids from *Escherichia coli* (Anagnostopoulos and Spizizen, 1961). Plasmids contained a chloramphenicol resistance gene (Band and Henner, 1984). *Bacillus* transformants were cultured 20–24 h in shake flasks containing LB media plus 12.5 μg/ml chloramphenicol at 37 °C.

To purify subtilisin, culture supernatants were dialyzed against 10 mM sodium phosphate, pH 6.2, for 12–20 h. Dialyzed broth was adjusted to pH 6.2 and loaded onto a CM52 column approximately one-tenth the volume of the dialyzed culture broth. After washing with 10 mM sodium phosphate, pH 6.2, the enzyme was eluted with the same buffer plus 0.08 M NaCl. The subtilisin peak was identified by enzyme activity and shown to be >95% pure by sodium dodecyl sulfate gel electrophoresis. Enzyme concentrations were determined spectrophotometrically ($\epsilon_{280}^{1\%}$ = 1.17; Matsubara *et al.*, 1965). The molar absorption coefficient for the tryptophan mutant was adjusted by a factor of 1.16 (Alan, 1981). No correction was made for small absorbance changes expected from the other aromatic amino acid substitutions. Enzymes were assayed in a solution containing 0.3 mM *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanalide (Vega Biochemicals), 0.1 M Tris, pH 8.6, at 25 °C. The assays measured the increase in absorbance at 410 nm/min due to hydrolysis and release of *p*-nitroanaline (ϵ_{410} = 8480 M^{-1} cm^{-1}; Del Mar *et al.*, 1979).

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The term "cassette mutagenesis" denotes the insertion of an oligodeoxynucleotide cassette (a synthetic double-stranded DNA fragment) into an appropriate plasmid.

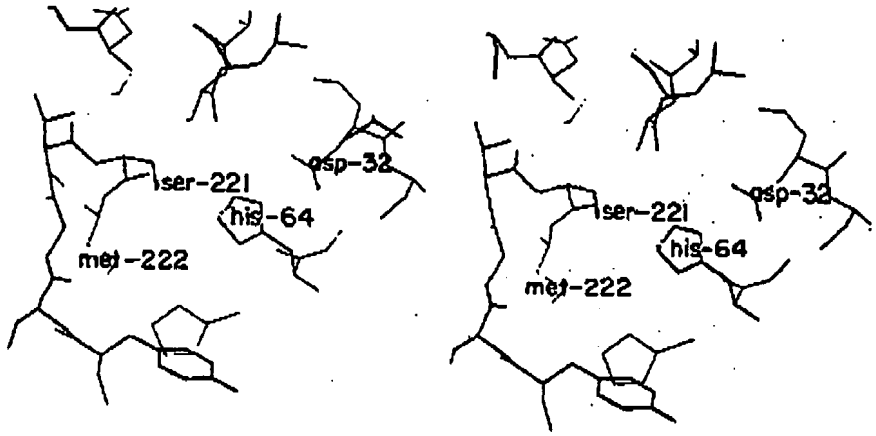

FIG. 1. **Stereoscopic view of the active site of subtilisin from *B. amyloliquefaciens* structure (Wright *et al.*, 1969).** Residues labeled include serine 221, histidine 64, and aspartate 32 which form the catalytic triad typical of serine proteases. Also labeled is methionine 222 which is the residue identified by peptide mapping studies to be oxidized to the sulfoxide by H_2O_2 with resultant inactivation (Stauffer and Etson, 1969). Substrate binds from the N to C terminus across the binding cleft extending from the upper right to lower left (Robertus *et al.*, 1972).

TABLE I

Relative specific activities of codon 222 mutant subtilisins

Mutant enzymes were purified and assayed as described under "Materials and Methods."

Codon 222	Relative specific activity
	%
Cys	138
Met	100
Ala	53
Ser	35
Gly	30
Thr	28
Asn	15
Pro	13
Leu	12
Val	9.3
Gln	7.2
Phe	4.9
Trp	4.8
Asp	4.1
Tyr	4.0
His	4.0
Glu	3.6
Ile	2.2
Arg	0.5
Lys	0.3

TABLE II

Kinetic constants for selected codon 222 mutants

K_m and V_{max} values were determined from initial rate measurements for hydrolysis of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanalide over a substrate concentration of 4×10^{-5} to 2.5×10^{-3} M in 0.1 M Tris, pH 8.6, at 25 °C (see "Materials and Methods"). Data were fit to the Michaelis-Menten equation using a nonlinear regression algorithm (Marquardt, 1963). Enzyme concentrations were determined spectrophotometrically to permit calculation of k_{cat} from the relationship of $k_{cat} = V_{max}/[\text{enzyme}]$.

Codon 222	K_{cat}	K_m	k_{cat}/K_m
	s^{-1}	M	$M^{-1}\,s^{-1}$
Met (wild-type)	50 (±1)	$1.4\ (\pm 0.05) \times 10^{-4}$	36×10^4
Cys	84 (±2)	$4.8\ (\pm 0.3) \times 10^{-4}$	20×10^4
Ser	27 (±1.8)	$6.3\ (\pm 0.6) \times 10^{-4}$	4×10^4
Ala	40 (±1)	$7.3\ (\pm 0.4) \times 10^{-4}$	5×10^4
Leu	5 (±0.1)	$2.6\ (\pm 0.2) \times 10^{-4}$	2×10^4

RESULTS AND DISCUSSION

Mutant subtilisin genes were expressed, and subtilisin was secreted from the *B. subtilis* strain BG2036. This strain contains deletions in the endogenous host subtilisin and neutral protease genes (Yang *et al.*, 1984) and so eliminates background secreted protease activity from these two major secreted proteases. Furthermore, possible recombination between the plasmid containing the mutant subtilisin gene and the host subtilisin gene can be avoided.

To determine the specific activity of each mutant enzyme, enzymes were purified from culture supernatants, and their concentrations were determined spectrophotometrically. The enzymes were assayed against the substrate, succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanalide, and relative specific activities *versus* wild-type (Met-222) are listed in Table I. There is a variation in specific activity of these mutants of 0.3–138% of wild-type. In general, small amino acids are the most active toward this substrate followed by the amino acids with amides and aliphatic side chains. Bulky aromatic and charged amino acid substitutions are less active. Position 222 is at the N terminus of an α-helix; thus, glycine and proline substitutions are accommodated reasonably well. Both sulfur-containing amino acids are most active, and the cysteine mutant has an even greater specific activity under these conditions than the wild-type enzyme.

To understand further the basis for variation in specific activity between mutant enzymes, the kinetic constants, k_{cat} and K_m, were determined for selected mutants. As shown in Table II, k_{cat} is greater for the cysteine mutant than for the wild-type. The specific activity data in Table I suggest that the cysteine mutant is a better enzyme than the methionine enzyme because at substrate saturation concentrations the comparison of these enzymes is weighted between the k_{cat} values. The increase in k_{cat} for cysteine 222 is coincident with a disproportionate increase in K_m as the catalytic efficiency of this enzyme is still below that of wild-type. Substrate binding is most significantly weakened in the cysteine, alanine, and serine mutants. However, for leucine 222, k_{cat} is seen to be lowered 10-fold with only a 2-fold increase in K_m. Studies are underway to determine the substrate dependence and structural basis for these altered functional parameters.

The mutant enzymes were evaluated for resistance to inactivation by 0.1 M H_2O_2. As shown in Fig. 2, the wild-type enzyme (methionine) was rapidly inactivated ($t_{1/2} \sim 2.5$ min). However, the serine and alanine enzymes were stable over a 1-h time course, as was leucine 222 (data not shown). Whereas the cysteine mutant was barely affected by 0.1 M H_2O_2, it was inactivated by 1 M H_2O_2 ($t_{1/2} \sim 12$ min) as shown in Fig. 2. The fact that the cysteine mutant oxidizes more slowly than methionine may reflect steric inaccessibility and/or intrinsic oxidation potential of the sulfur. The activity of the serine and alanine mutants remains unaffected by 1 M H_2O_2. When the wild-type enzyme was added to the serine-substituted

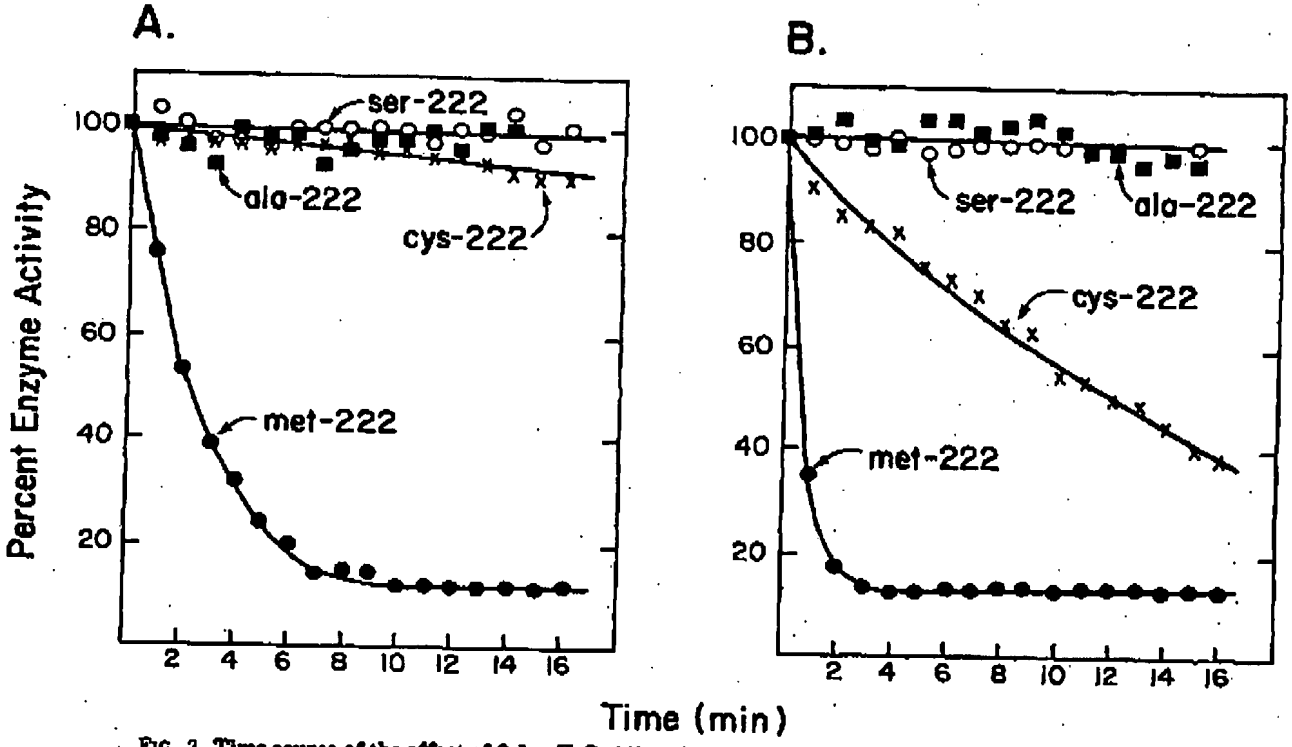

FIG. 2. Time course of the effect of 0.1 M H_2O_2 (*A*) or 1.0 M H_2O_2 (*B*) on the activity of purified wild-type and codon 222 mutant subtilisins. Mutant and wild-type enzymes were incubated in the presence of fresh H_2O_2 (from a fresh 30% stock bottle) and 0.1 M sodium borate, pH 9.5. At the indicated times, reactions were quenched by dilution of enzyme into assay mixture containing 0.3 mM *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanalide, 0.1 M Tris, pH 8.6. Residual activity is expressed as a per cent of a nontreated enzyme control.

enzyme after 1 h of incubation with 1 M H_2O_2, the former was rapidly inactivated, indicating the continued presence of H_2O_2 (data not shown).

The oxidized wild-type enzyme, which Stauffer and Etson (1969) showed to be methionine sulfoxide, maintains approximately 12% residual activity when in either 0.1 or 1 M H_2O_2. This suggests that the oxidized enzyme is still 12% active. Similarly, the cysteine enzyme maintains approximately 7% residual enzyme activity after prolonged treatment (45 min) with 1 M H_2O_2 (data not shown). Under these conditions, the cysteine should have oxidized to the sulfonate (Means and Feeney, 1971). It is interesting to note that the oxidized derivatives of methionine and cysteine (*i.e.* methionine sulfoxide and cysteine sulfonate, respectively) are steric and charged homologues of glutamine and aspartic acid, respectively; these derivatives possess about the same corresponding specific activity. Thus, whether introduced by mutagenesis or by chemical modification, bulky or charged substitutions at position 222 have a deleterious effect on enzyme function.

Stauffer and Etson (1969) showed that the generation of methionine sulfoxide by 0.1 M H_2O_2 correlated with the reduction of enzyme activity. However, their data on total amino acid composition during H_2O_2 treatment could not rigorously exclude the possible oxidation of other activity critical residues. The data presented here show that substitution of methionine 222 by an oxidatively stable residue (*i.e.* Ala, Ser) imparts oxidative resistance as measured by enzyme activity. Although other sites may be oxidized, it is clear from these data that substitutions at position 222 affect catalytic efficiency and oxidative stability. The alanine and serine mutants appear to be the optimal derivatives for combined specific activity against *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanalide and oxidative resistance.

Predictions of optimal amino acid replacement based on homologous exchange data (Dayhoff *et al.*, 1978) would have incorrectly targeted leucine and valine as optimal methionine substitutes. Because of uncertainties in structure-function relationships, multiple amino acid replacements were required to find optimal methionine substitutes. This work and that of Rosenberg *et al.* (1984)[2] demonstrate that oxidative stability in these proteins can be improved by replacement of oxidatively sensitive residues which are activity critical.

Chemical oxidation can be a significant source of enzyme inactivation particularly for enzymes which function extracellularly (Brot and Weissbach, 1983). Even though oxidatively sensitive residues may be of functional importance, this work demonstrates that it may be possible to find oxidatively stable amino acid substitutions which maintain enzyme function. This could have significant benefit in stabilizing enzymes used in industrial processes. We speculate that enzymes may be produced which are resistant to inactivating chemical modifications at noncatalytic residues by substitution for amino acids which are resistant to such modifications.

Acknowledgments—We wish to thank Dr. Rick Bott for help with computer graphics. We are grateful to the support of Dr. William Rastetter, Dr. Rey Gomez, and the Protein Engineering efforts at Genentech and Genencor. Drs. Steve Anderson, Tony Kossiakoff, and Ron Wetzel provided constructive comments on this manuscript.

[2] At the time the present work was submitted, this group showed that replacement of a methionine residue with valine in the active site of α_1-anti-trypsin inhibitor resulted in a functional inhibitor having greater oxidative stability. No other mutants were described.

REFERENCES

Alan, G. (1981) *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 9, p. 27, Elsevier/North-Holland, New York

Anagnostopoulos, C., and Spizizen, J. (1961) *J. Bacteriol.* **81**, 741–746

Band, L., and Henner, D. J. (1984) *Gene (Amst.)* **3**, 17–21

Brot, N., and Weissbach, H. (1983) *Arch. Biochem. Biophys.* **223**, 271–281

Brot, N., Weissbach, L., Werth, J., and Weissbach, H. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 2155–2158

Dayhoff, M. O., Schwartz, R. M., and Orcutt, B. C. (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., ed) Vol. V, Supplement 3, pp. 345–352, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D. C.

DelMar, E. G., Largman, C., Brodrick, J. W., and Geokas, M. C. (1979) *Anal. Biochem.* **99**, 316–320

Drenth, J., Hol, W. G. J., and Jansonius, J. (1972) *Eur. J. Biochem.* **26**, 177–181

Markland, F. S., and Smith, E. L. (1971) in *The Enzymes* (Boyer, P. D., ed) Vol. III, pp. 561–608, Academic Press, New York

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math* **11**, 431–441

Matsubara, H., Kasper, C. B., Brown, D. M., and Smith, E. L. (1965) *J. Biol. Chem.* **240**, 1125–1130

Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins*. pp. 162–165, Holden-Day, Inc., Oakland, CA

Robertus, J. D., Kraut, J., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C., and Wilcox, P. E. (1972) *Biochemistry* **11**, 2439–2449

Rosenberg, S., Barr, P. J., Najarian, R. C., and Hallewell, R. A. (1984) *Nature* **312**, 77–80

Stauffer, C. E., and Etson, D. (1969) *J. Biol. Chem.* **244**, 5333–5338

Wallace, R. B., Schold, M., Johnson, M. J., Dembek, P., and Itakura, K. (1981) *Nucleic Acids Res.* **9**, 3647–3656

Wells, J. A., Ferrari, E., Henner, D. J., Estell, D. A., and Chen, E. Y. (1983) *Nucleic Acids Res.* **11**, 7911–7924

Wells, J. A., Vasser, M., and Powers, D. P. (1985) *Gene (Amst.)* in press

Wright, C. S., Alden, R. A., and Kraut, J. (1969) *Nature* **221**, 235–242

Yang, J., Ferrari, F., and Henner, D. J. (1984) *J. Bacteriol.* **160**, 15–21

Zoller, M. J., and Smith, M. (1982) *Nucleic Acids Res.* **10**, 6487–6500